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Paeoniflorin Prevents Diabetic Nephropathy in Rats

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The aim of this study was to test the hypothesis that paeoniflorin prevents the progression of diabetic nephropathy by modulating the inflammatory process. Sprague–Dawley rats were divided into 5 groups: nondiabetic control rats; untreated diabetic model (DM) rats; and DM rats treated with 5, 10, or 20 mg/kg paeoniflorin in drinking water once daily. Rats received a single intravenous injection of streptozotocin to induce diabetes; 9 wk after injection, rats began the 8-wk daily paeoniflorin treatment regimen. Compared with that of nonDM controls, the urinary albumin:creatinine ratio was increased significantly in untreated DM rats; this ratio was decreased in DM rats treated with 5, 10, or 20 mg/kg paeoniflorin compared with that of untreated DM rats. In addition, paeoniflorin treatment effectively suppressed glomerular hypertrophy; blood glucose; the expression of transforming growth factor β, type IV collagen, and intercellular adhesion molecule 1; and renal infiltration of macrophages compared with levels in untreated DM rats. Furthermore, renal nuclear factor κB activity was increased in untreated but not paeoniflorin-treated DM rats. In conclusion, our data suggest that the preventive effects of paeoniflorin may be mediated by its antiinflammatory actions.

Abbreviations: DM, diabetic model; ECM, extracellular matrix; ICAM1, intercellular adhesion molecule 1; MCP1, monocyte chemoattractant protein 1; NFκB, nuclear factor κB; TGFβ, transforming growth factor β.

Diabetic nephropathy is the most common cause of endstage renal disease and high mortality in humans. Adequate control of blood glucose may slow the rate of its progression, but it is still difficult to achieve strict glycemic control for diabetic patients in the longer term, due at least in part to the limitations of available therapeutic approaches.[3] Recent studies have suggested the emerging role of inflammatory processes in the pathogenesis of diabetic nephropathy in addition to other well-known mechanisms.

In human renal disease, transforming growth factor β (TGFβ) may mediate the buildup of tissue extracellular matrix (ECM) proteins.[17] This cytokine reportedly stimulated ECM protein accumulation in diabetic tissues by upregulating the production of ECM proteins or by downregulating the production of ECM-degrading enzymes.[26] Renal levels of TGFβ1 increase in both experimental and human diabetes. In addition, TGFβ1 induces the synthesis of ECM components including collagen types I, III, and IV and fibronectin.[2,5]

Intercellular adhesion molecule 1 (ICAM1) is a key adhesion molecules. In addition, the ICAM1-dependent infiltration of macrophages into the kidney is very important in the pathogenesis of diabetic nephropathy.[18] In addition, the expression of ICAM1 is rapidly induced and maintained for a long time in renal tissues after induction of diabetes in experimental type 1 diabetic rats.[14,20] Macrophage infiltration was blocked by antiICAM1 antibody, confirming that ICAM1 mediates macrophage infiltration into the diabetic kidney.[6] Furthermore, ICAM1-deficient mice were protected from renal injury after the induction of diabetes, suggesting that the inflammatory process is a critical factor for the development of diabetic nephropathy.[21]

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Despite the availability of treatments that lower blood glucose and blood pressure, many diabetic patients are still prone to developing kidney failure, which no currently available therapies can reverse.[24] Therefore a search is needed for new therapeutic approaches—based on novel mechanisms of action—to the treatment of diabetic nephropathy. Paeoniflorin is a monoterpene glucoside and a component of the total glucoside extract obtained from the root of *Paeonia lactiflora*.[28] This extract was approved for marketing in China in 1998.[23] As a disease-modifying drug, the total glucoside extract of peony has both antiinflammatory and immune-regulatory effects and is used in the treatment of rheumatoid arthritis, hepatitis, systemic lupus erythematosus, and mesenteric hyperplastic nephritis.[8,9,27] The goal of this study is to address whether paeoniflorin might prevent the progression of diabetic nephropathy through the inhibition of the inflammatory processes including TGFβ, type IV collagens, and ICAM1 expression, monocyte chemoattractant protein 1 (MCP1), nuclear factor κB (NFκB) activation, and macrophage infiltration.

Materials and Methods

Materials. ***Animals.*** Animals were housed in facilities conforming to international guidelines,[9] and the studies were approved by our institutional animal care and use committee.

Female Harlan Sprague–Dawley pathogen-free rats ($n = 50$; age, 5 wk; weight, 220 to 250 g; Experimental Animal Center of Fourth Military Medical University, Xi, China) were housed 5 per cage with free access to food and water and were kept in a constant environment (22 ± 2 °C, 50% ± 5% humidity, 12:12-h light:dark cycle). These rats were divided into 5 groups of 10 rats each: nondiabetic (nonDM) control rats; untreated diabetic (DM) rats; and DM rats treated with 5, 10, or 20 mg/kg paeoniflorin daily.

Chemicals and reagents. Paeoniflorin ($C_{23}H_{28}O_{11}$; molecular weight, 480.45; purity, at least 95% (by HPLC); LD_{50}, 9530 mg/kg; Xuancheng Baicao Plants Industry and Trade, Anhui, China)

Table 1. Metabolic data at 17 wk after induction of diabetes

			Paeoniflorin dose (mg/kg PO daily)		
	nonDM	DM	5	10	20
Body weight (g)	388.65 ± 10.82	309.24 ± 11.83[a]	301.12 ± 10.97[a]	299.06 ± 10.29[a]	297.34 ± 9.87[a]
Kidney weight (g)	1.58 ± 0.14	2.09 ± 0.20[a]	1.82 ± 0.18[a]	1.73 ± 0.15	1.59 ± 0.11
Blood pressure (mmHg)	110.06 ± 2.19	117.25 ± 3.56	115.98 ± 3.42	114.71 ± 2.64	113.32 ± 2.07
Blood glucose (mg/dL)	142.19 ± 18.33	525.21 ± 20.38[b]	419.36 ± 22.17[b,c]	302.46 ± 19.35[a,d]	228.62 ± 23.90[a,d]
Urinary albumin:creatinine ratio	112.41 ± 31.78	498.07 ± 58.32[b]	311.69 ± 40.76[b,c]	268.34 ± 38.08[a,d]	204.52 ± 35.71[a,d]

Data are presented as mean ± 1 SD (n = 10 rats per group).
[a]$P < 0.05$ compared with value for nonDM rats.
[b]$P < 0.001$ compared with value for nonDM rats.
[c]$P < 0.05$ compared with value for DM rats.
[d]$P < 0.01$ compared with value for DM rats.

was dissolved in distilled water. All chemicals were of analytical reagent grade. Before the experiment, all of the vessels and tips for pipetting were dipped in strong HNO_3 for 24 h and then washed with ultrapure water. The water used was purified by electrodeionization (Milli-Q Water Purification System, Millipore, Bedford, MA).

Modeling. Rats were rendered diabetic by a single intravenous injection of streptozotocin (65 mg/kg body weight; Sigma–Aldrich, St Louis, MO) in 1 M sodium citrate buffer (pH 4.5) as described previously.[6] Body weights and blood glucose values were monitored at 3 and 7 d after injection, and only rats with blood glucose concentrations greater than 16 mmol/L were used in the study. Beginning 9 wk after streptozotocin injection, paeoniflorin (5, 10, or 20 mg/kg) was administered daily in the drinking water (18 MΩ) for 8 wk. At 17 wk after diabetes induction, rats were anesthetized and euthanized by CO_2 overdose. Both kidneys were excised immediately, weighed, and fixed in 10% formalin for periodic acid–Schiff staining. The remaining tissues were embedded in Optimal Cutting Temperature compound (Sakura Fine Technical, Tokyo, Japan) and immediately frozen in acetone cooled on dry ice. For electrophoretic mobility shift assays, tissues were snap-frozen in liquid nitrogen and stored at –80 °C.

Metabolic data analysis. At 17 wk after diabetes induction, we measured body weight, kidney weight, blood pressure, blood glucose levels, 24-h urinary albumin excretion, and creatinine clearance. Blood pressure was measured by using the tail-cuff method.[10,18] Rats were placed individually in metabolism cages (TSE Systems, Midland, MI), 24-h urine samples were collected in metal-free propylene tubes (Nalge, Rochester, NY), and 24-h urinary albumin excretion was determined by immunoassay (Rat Albumin Enzyme Immunoassay; SPI-BIO, Montreal, Quebec, Canada). The urinary albumin:creatinine ratio was calculated from these 2 measurements. Creatinine clearance was calculated on the basis of urinary creatinine, serum creatinine, urine volume, and body weight by using the following equation:

Creatinine clearance = [urinary creatinine (μmol/L) × 24-h urine volume (mL)] / {serum creatinine (μmol/L) × [1000 / body weight (g)] × [1 / 86,400 s]}.

Histologic analysis. Light microscopy of periodic acid–Schiff-stained kidney sections (thickness, 4 μm) was used for morphometric studies. To evaluate glomerular size, 100 glomeruli were evaluated for each group. The glomerular surface area (μm^2) was determined from digital images by a single blinded observer using the Soft Imaging System (Olympus, London, United Kingdom).

Immunohistochemical and immunoperoxidase staining. For immunohistochemical and immunoperoxidase staining, tissues were fixed in 10% buffered formalin and embedded in paraffin. Commercially available monoclonal antibodies to TGFβ (catalog no., sc52829; Santa Cruz Biotechnology, Santa Cruz, CA), type IV collagen (catalog no., sc52317; Santa Cruz Biotechnology), ICAM1 (catalog no., sc71299; Santa Cruz Biotechnology) and monocytes–macrophages (ED1; catalog no., sc59103; Santa Cruz Biotechnology) were used. Samples were stained by using the avidin–biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA).

A single paraffin-embedded block of tissue was selected from each animal and cut into 4-μm sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 min before conducting antigen retrieval by using a microwave oven at 95 °C for 5 min and cooling at 25 °C for 2 h. After sections were washed with PBS, blocking serum was applied for 10 min, after which the sections were incubated with antimouse monoclonal antibodies to TGFβ (1:100), type IV collagen (1:50), ICAM1 (1:100), and monocytes–macrophage (1:150; all antibodies from Santa Cruz Biotechnology) overnight at 4 °C, respectively. After sections were washed in PBS, biotin-labeled secondary antibody was applied for 10 min followed by peroxidase-conjugated streptavidin for an additional 10 min. The reaction was visualized by using 3, 3′-diaminobenzidine tetrahydrochloride. Nuclei were counterstained with hematoxylin, and positive and negative immunohistochemistry controls were included. Reproducibility of staining was confirmed by immunostaining multiple, random specimens by the same method.

TGFβ, type IV collagen, and ICAM1 immunostaining intensities were quantified by a modified method.[20] The brightness of each image file was enhanced uniformly by using Photoshop (Adobe, San Jose, CA), followed by analysis by using Scion Image software (Scion Corporation, Frederick, MD). TIFF image files were inverted and opened in grayscale mode. The TGFβ, type IV collagen, and ICAM1 indices were calculated by using the formula

[staining density × positive area (μm^2)] / total glomerular area (μm^2),

where the staining density is indicated by a number from 100 to 256 in grayscale.

To evaluate the infiltration of macrophages, intraglomerular ED1-positive cells were counted in 200 glomeruli per group by 2 independent observers with no prior knowledge of the exper-

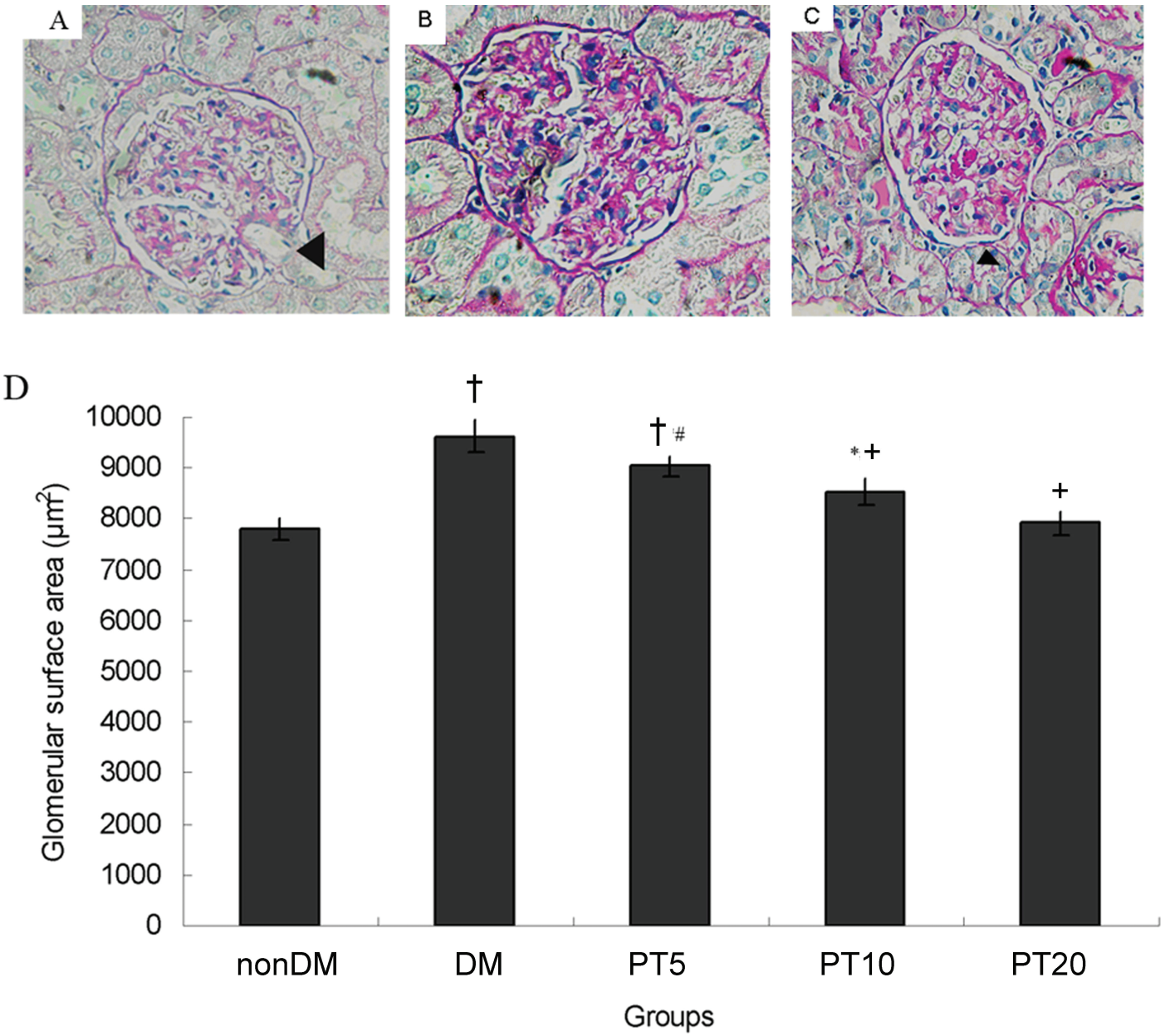
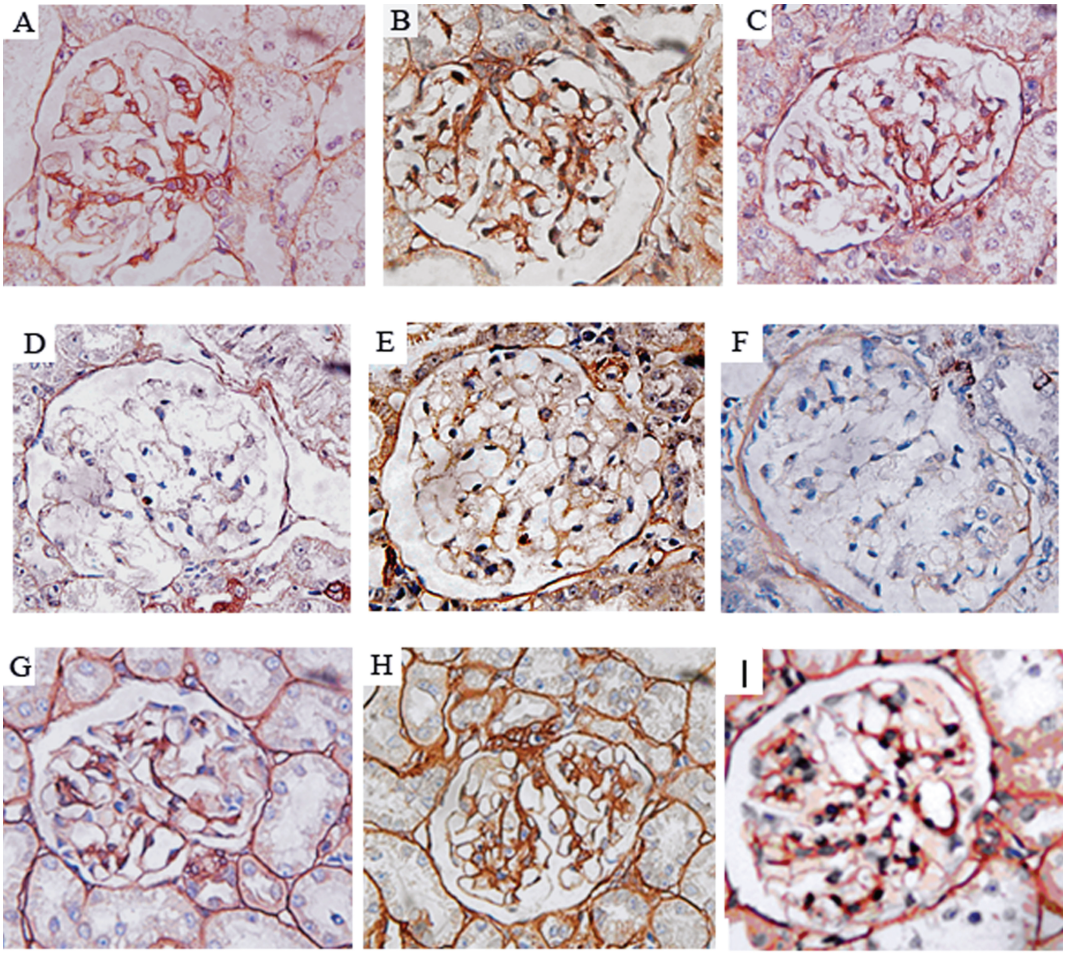


Figure 1. Effect of paeoniflorin treatment on glomerular hypertrophy in diabetic rats. Representative photomicrographs of kidney sections stained with periodic acid–Schiff from (A) nonDM, (B) DM, and (C) paeoniflorin-treated (20 mg/kg daily) rats. Original magnification, ×400. (D) Glomerular surface area in nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. Glomerular cross-sectional area was measured from kidney sections stained with periodic acid–Schiff. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

imental design. The average number of ED1-positive cells per glomerulus was used for estimation.

Western blotting. Kidney samples from each of the 5 groups were homogenized in lysis buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate at 4 °C throughout all procedures and sonicated for 70 s, after which 300 μg phenylmethylsulfonyl fluoride was added per gram of tissue and the homogenate incubated on ice for 30 min, followed by centrifugation at 4892.37 × *g* for 20 min at 4 °C. The protein content was determined according to Bradford method,[1] with bovine serum albumin used as a standard. Protein samples (30 μg) were boiled for 5 min with 2× sample buffer containing 5% β-mercaptoethanol, separated by size on 15% polyacrylamide gel under SDS denaturing conditions, and transferred to a nitrocellulose membrane at 90 V for 2 h.

The nitrocellulose membranes were stained with Ponceau S to assess the efficiency of transfer. The membranes were blocked with 5% nonfat dry milk in 20 mM Tris-buffered saline at room temperature for 2 h and incubated with polyclonal rabbit antirat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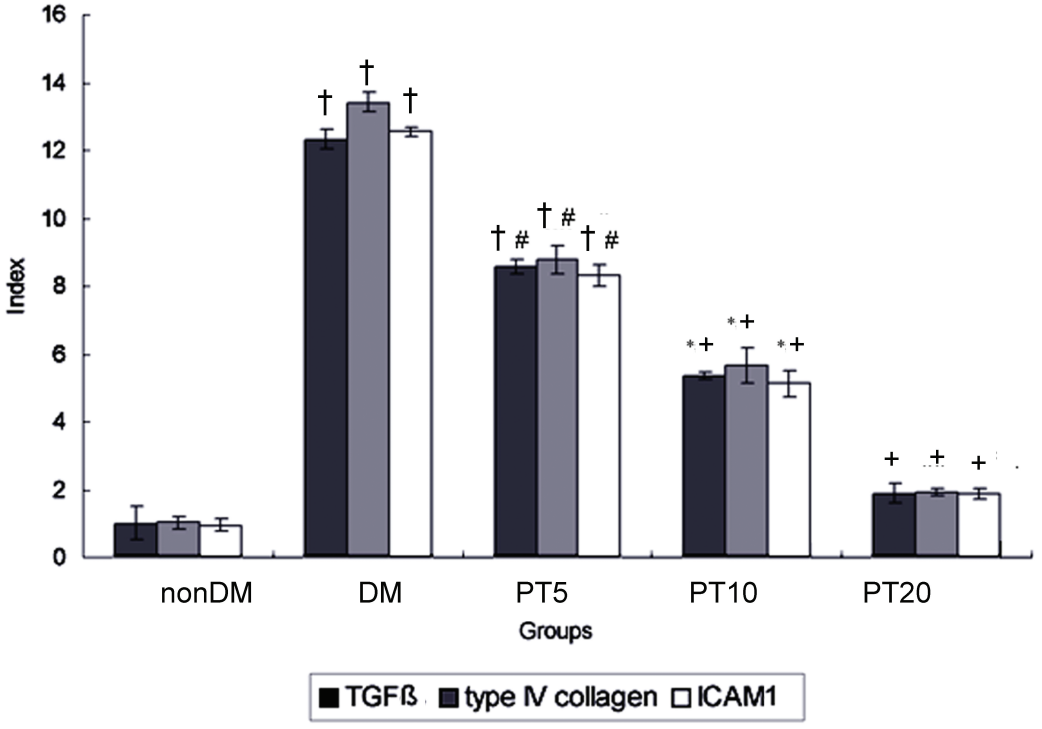
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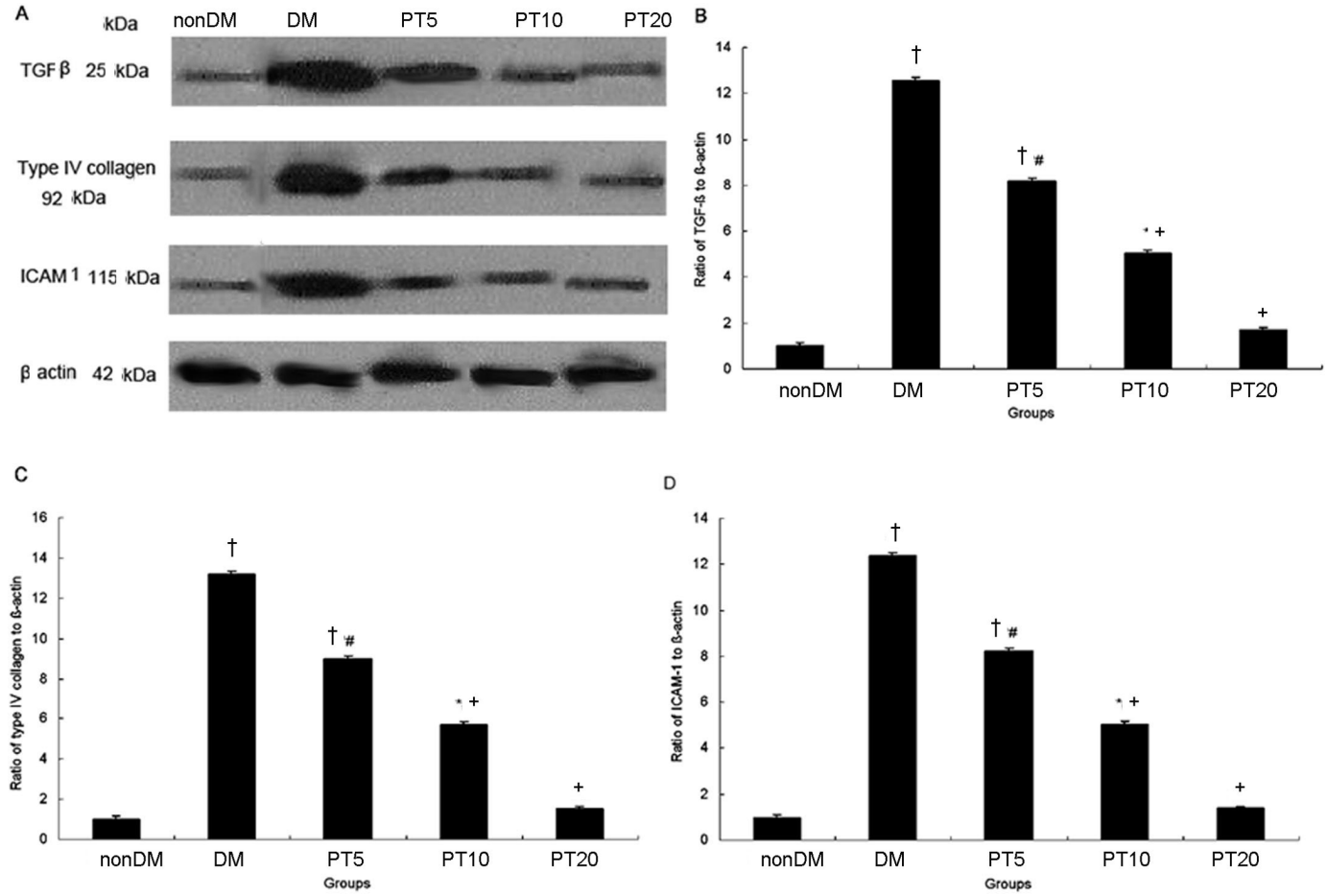

Figure 3. Photograph of gel from Western blot analysis (A) and densitometric analysis of TGFβ protein (B), type IV collagen protein (C), and ICAM1 protein (D) in kidney tissues of nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

antibodies to TGFβ (1:1000), type IV collagen (1:500), and ICAM1 (1:500; all antibodies from Santa Cruz Biotechnology) at 4 °C overnight, and then incubated with horseradish peroxidase-labeled goat antirabbit IgG (1:500). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Life Science, Little Chalfont, UK). The housekeeping protein β-actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Q500IW Image Analysis System, Leica Microsystems, Bannockburn, IL) and expressed as the ratio of TGFβ, type IV collagen, and ICAM1 to β-actin in optical density units.

Real-time RT-PCR. The mRNA expression of TGFβ, type IV collagen, ICAM1, and β-actin (as an internal control) in kidney from each of the 5 groups was determined by using real-time RT-PCR. Total RNA was isolated from kidney tissue by the guanidinium–acid phenol method (Isogen kit, Nippon Gene, Tokyo, Japan), and the concentration of RNA was determined by absorbance at 260 nm in relation to that at 280 nm. The isolated RNA was stored at –70 °C until use. Extracted RNA (1 mg) was reverse-transcribed into first-strand cDNA at 42 °C for 40 min by using 200 U MMLV reverse transcriptase (Promega, Madison, WI) and 0.5 mg oligo(dT)$_{15}$ primer (Takara Bio, Shiga, Japan) in a 20-μL reaction mixture. Real-time RT-PCR for TGFβ, type IV collagen, ICAM1, and β-actin was carried out (ABI7300 System, Applied Biosystems, Foster City, CA) by using the DNA-binding dye SYBER Green I for the detection of PCR products. The reaction mixture contained 12.5 μL Premix Ex *Taq* and 0.5 μL ROX reference dye (RRO41A, Takara Bio), 1 μL custom-synthesized primers, and 2 μL cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μL. Primer sequences (size of amplicon)

Figure 2. Expression of TGFβ, type IV collagen, and ICAM1 in kidney tissues of rats. TGFβ expression (black bars) in (A) nonDM, (B) DM, and (C) paeoniflorin-treated (20 mg/kg daily; PT20) rats. Type IV collagen expression (gray bars) in (D) nonDM, (E) DM, and (F) PT20 rats. ICAM1 expression (white bars) in (G) nonDM, (H) DM, and (I) PT20 rats. (J) Immunostaining intensity for TGFβ, type IV collagen, and ICAM1 in glomeruli of rats in in nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily) rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

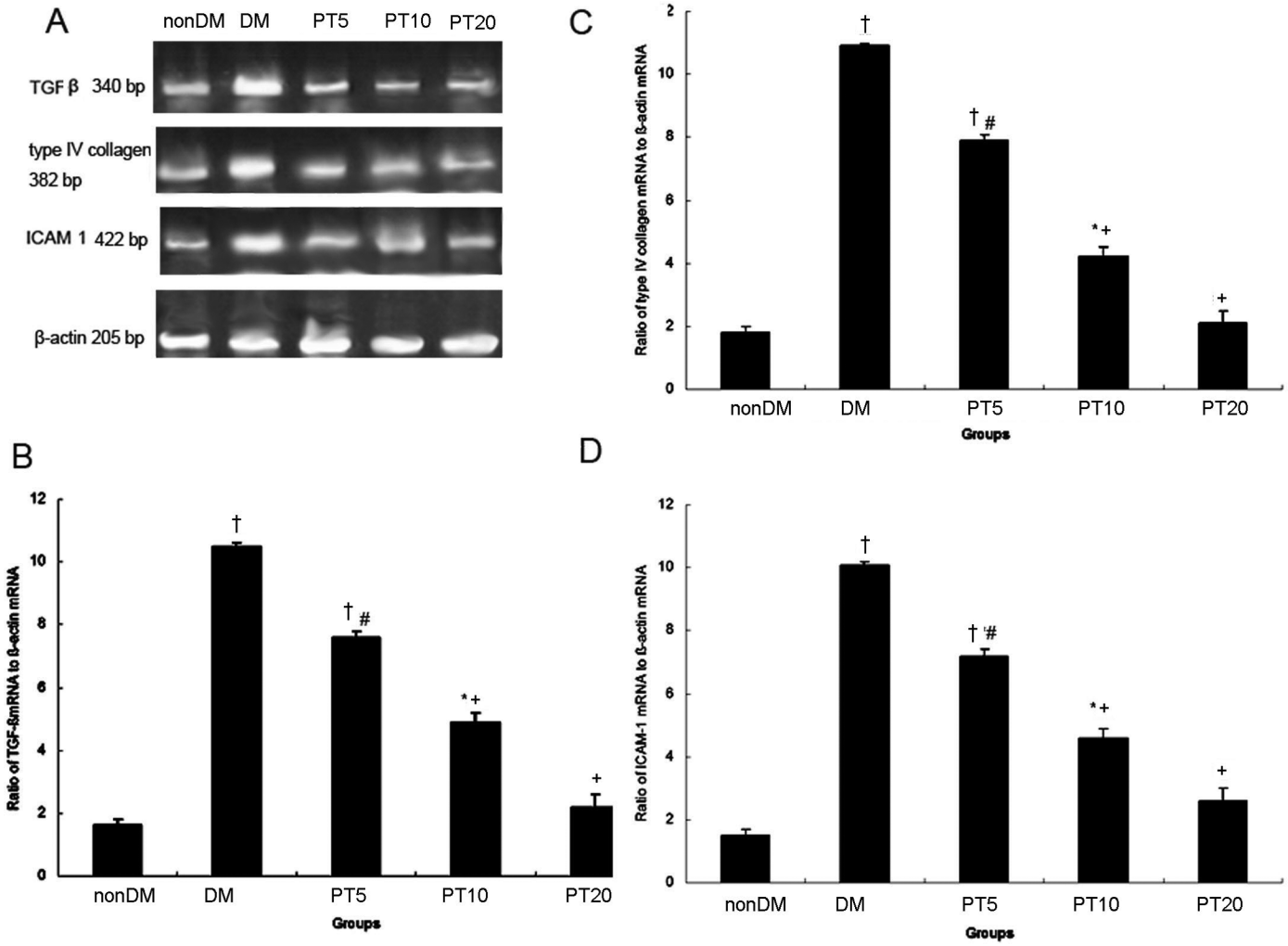

Figure 4. Photograph of gel from (A) real-time RT-PCR analysis and densitometric analysis of (B) TGFβ protein, (C) type IV collagen protein, and (D) ICAM1 protein in kidney tissues of nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

were: TGFβ (340 bp): sense 5′ GCT AAT GGT GGA CCG CAA C 3′ and antisense 5′ GCA GTG AGC ACT GAA GCG A 3′; type IV collagen (382 bp): sense 5′ ATG TCA ATG GCA CCC ATC AC 3′ and antisense 5′ CTT CAA GGT GGA CGG CGT AG 3′; ICAM1 (422 bp), sense 5′ CGT GCT GTA TGG TCC TCG 3′ and antisense 5′ GGG CTT GTC CCT TGA GTT 3′; and β-actin (205 bp), sense 5′ CCT TCC TGG GCA TGG AGT CCT G 3′ and antisense 5′ GGA GCA ATG ATC TTG ATC TTC 3′. The PCR conditions were: initial denaturation for 15 s at 95 °C; 40 cycles of 95 °C for 15 s, 60 °C for 31 s. Real-time RT-PCR data were used to calculate expression of mRNA from target gene relative to that of β-actin.

Serum levels of MCP1 in different groups. The serum level of MCP1 in each of the 5 groups was determined by using an ELISA kit (Endogen, Cambridge, MA). The assays were performed according to the manufacturer's instructions, and all samples were analyzed at a dilution resulting in concentrations within the range of the standard curve. The limit of detection was 1 pg/mL; levels below 1 pg/mL were considered undetectable.

NFκB activity assay. Electrophoretic mobility shift assays were performed to analyze NFκB activation. Briefly, frozen kidney cortical sections were minced, suspended in 1 mL 20 mM Tris-buffered saline, and homogenized. The homogenates were centrifuged, and the pellets were resuspended in 1 mL buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] and chilled on ice for 20 min. Next, 100 µL 10% NP40 was added and the solution vortexed vigorously. The nuclear fraction was collected by centrifugation and resuspended in 100 µL buffer B [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride]. For electrophoretic mobility shift assays, 50 µL nuclear extract were incubated with 1 ng (γ-^{32}P)ATP-labeled oligonucleotide containing an NFκB binding site (5′ AGT TGA GGG GAC TTC CCA GGC 3′) in 20 µL binding buffer [10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol, and 50 mg/mL dI-dC] for 30 min at room temperature. Individual samples were electrophoresed on a 4% polyacrylamide gel. The gel was dried

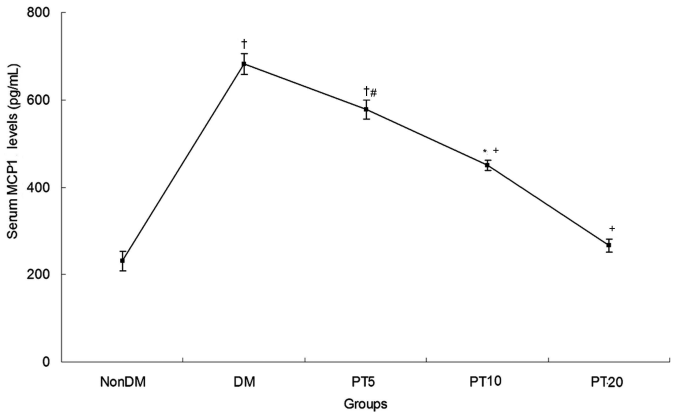

Figure 5. Serum MCP1 concentration of nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

and exposed to X-ray film (Hyperfilm MP, Amersham). The relative intensity of the autoradiogram was determined by using a scanning densitometer. Each experiment was performed 3 times.

Statistical analysis The software of SPSS version 16.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis. The data obtained are expressed as mean ± 1 SD and analyzed by 1-way ANOVA with post hoc Tukey tests for paired comparisons. A difference between means was considered significant if the P value was less than 0.05.

Results

Paeoniflorin decreased the urinary albumin:creatinine ratio in diabetic rats. At 17 wk after induction of diabetes, untreated diabetic (DM) rats exhibited a significant increase in urinary albumin:creatinine ratio when compared with nondiabetic controls (nonDM; 498.07 ± 58.32 μg/mg versus 112.41 ± 31.78 μg/mg, $P < 0.001$). Paeoniflorin decreased ($P < 0.01$) the urinary albumin:creatinine ratio in diabetic rats in a dose-dependent manner (Table 1). Therefore, paeoniflorin significantly suppressed the development of albuminuria in diabetic rats. Systolic blood pressure did not differ among the 5 groups. The initial blood glucose levels of the DM and all paeoniflorin-treated (PT) diabetic rats were significantly ($P < 0.05$) higher than that of the nondiabetic control group (Table 1), and paeoniflorin treatment efficiently reduced the blood glucose levels of diabetic rats (PT groups versus DM group, $P < 0.05$; Table 1).

Paeoniflorin attenuated renal and glomerular hypertrophy in diabetic rats. At 17 wk after induction of diabetes, rats in the DM and PF groups weighed significantly ($P < 0.05$) less than those in the nonDM group. Untreated diabetes caused renal hypertrophy characterized by significantly ($P < 0.05$) higher kidney weight in the DM rats (2.09 ± 0.20 g) compared with nonDM controls (1.58 ± 0.14 g). Renal hypertrophy was ameliorated dramatically by daily paeoniflorin treatment at doses of 10 mg/kg or more (PF10 versus nonDM, $P > 0.05$).

We next determined whether paeoniflorin-evoked amelioration of renal hypertrophy in diabetic rats was accompanied by a similar attenuation of glomerular hypertrophy. Glomeruli from untreated diabetic (DM) rats tended to be larger ($P < 0.05$) than those from nonDM controls, whereas glomerular surface area was reduced ($P < 0.05$) in a dose-dependent manner after paeoniflorin treatment (PT groups) compared with DM rats (Figure 1). Collectively, these results indicate that paeoniflorin attenuated both renal hypertrophy and glomerular hypertrophy in diabetic animals.

Paeoniflorin suppressed the expression of TGFβ, type IV collagen, and ICAM1. DM rats had higher renal TGFβ expression than did nonDM rats. Paeoniflorin treatment reduced renal TGFβ expression ($P < 0.001$) and type IV collagen ($P < 0.001$) in diabetic rats in a dose-dependent manner. ICAM1 expression was enhanced significantly ($P < 0.001$) in DM rats compared with nonDM rats, and paeoniflorin suppressed the increase in protein at 17 wk after the induction of diabetes ($P < 0.05$, Figure 2).

Densitometric analysis of Western blots showed 12.58-, 12.96-, and 12.89-fold increases in the amounts of TGFβ, type IV collagen, and ICAM1, respectively, in DM rats compared with nonDM rats; treatment with paeoniflorin significantly decreased TGFβ, type IV collagen, and ICAM1 protein expression (all $P < 0.05$; Figure 3). Real-time RT-PCR confirmed the results obtained by Western blotting (Figure 4).

Serum levels of MCP1 in different groups. The concentration of MCP1 in DM rats was significantly higher ($P = 0.008$) than that of nonDM rats. However, MCP1 was decreased markedly ($P < 0.05$) in a dose-dependent manner in the paeoniflorin-treatment groups compared with the DM group (Figure 5).

Paeoniflorin reduced glomerular infiltration of macrophages. The number of macrophages (ED1-positive cells) in glomeruli was significantly ($P < 0.05$) higher in the DM group than in the nonDM group. Paeoniflorin treatment reduced the number of glomerular infiltrating macrophages in a dose-dependent manner ($P < 0.05$, Figure 6).

Paeoniflorin reduced NFκB activation in the renal cortex. To analyze the renal binding activity of NFκB DNA, electrophoretic mobility shift assays were performed, the results of which are shown as relative intensity (Figure 7). NFκB activity was increased in the kidneys of rats in the DM group (relative intensity, 3.06 ± 0.32) compared with that in the non-DM group (1.08 ± 0.21, $P < 0.05$). Daily paeoniflorin treatment at doses of 10 mg/kg or higher significantly ($P < 0.05$) reduced the renal DNA-binding activity of NFκB compared with that in the DM group.

Discussion

Diabetic nephropathy is a long-term complication of diabetes, and effective blockade of the progression of nephropathy remains a medical challenge. In the present study, we showed that treatment with paeoniflorin ameliorated albuminuria and glomerular hypertrophy with reducing blood glucose levels in streptozotocin-induced diabetic rats—findings that were consistent with a previous study.[7] Perhaps more importantly, paeoniflorin also suppressed diabetes-induced increases in the urinary albumin:creatinine ratio, a key measure of renal function, in diabetic rats. Paeoniflorin treatment markedly and dose-dependently decreased TGFβ, type IV collagen, and ICAM1 mRNA and protein expression in diabetic kidneys and macrophage infiltration in diabetic glomeruli. In addition, paeoniflorin inhibited serum MCP1 in diabetic nephropathy rats. Furthermore, we found that paeoniflorin suppressed the activity of NFκB, a key transcription factor of many proinflammatory genes. Our findings suggest that

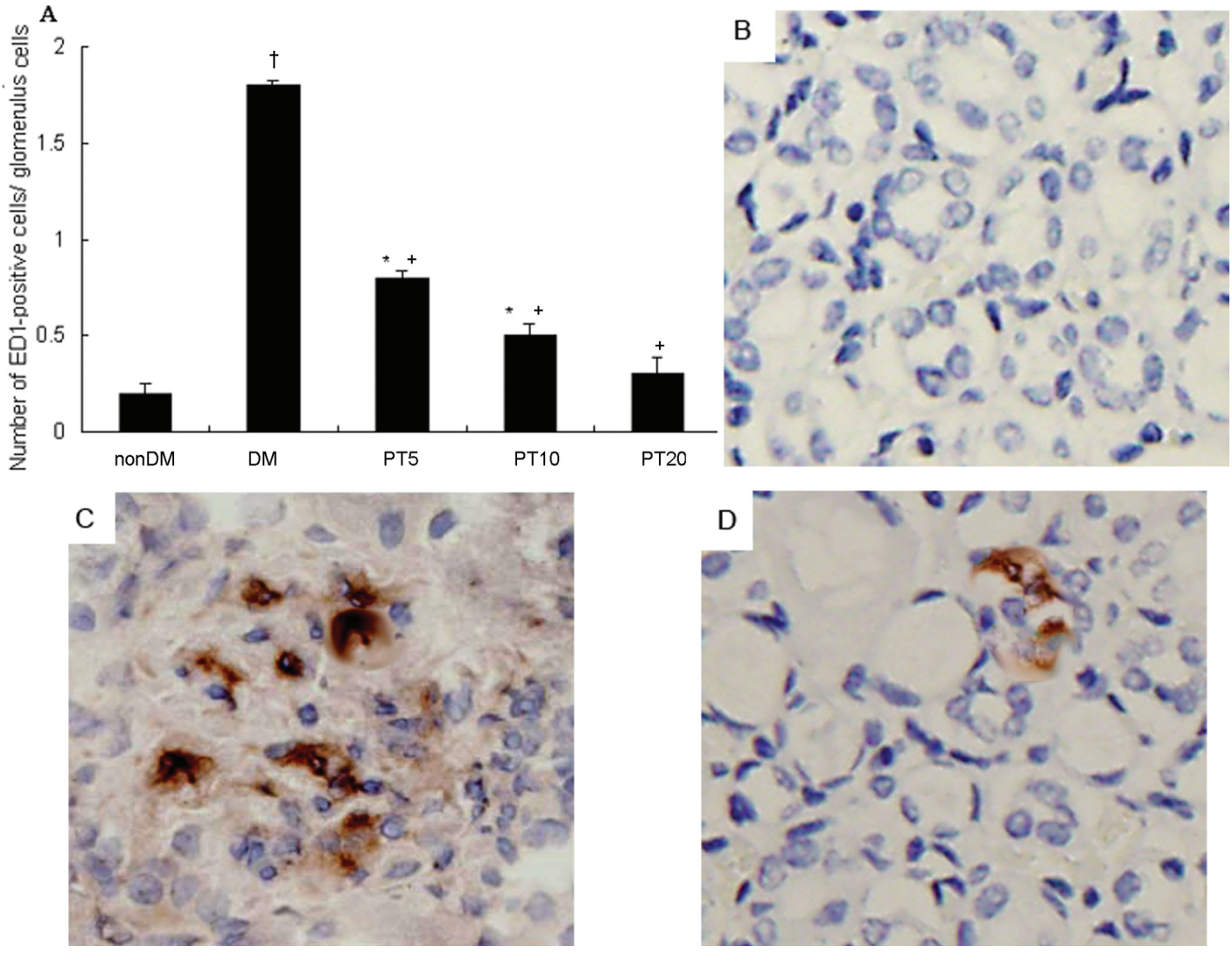

Figure 6. (A) Number of macrophages (ED1-positive cells) in the glomeruli of nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. Immunoperoxidase staining for macrophages (ED1-positive cells) in glomeruli of (B) non DM, (C) DM, and (D) PT20 rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests.

paeoniflorin has an antiinflammatory effect in diabetic kidneys and prevents the development of nephropathy.

Peony plants, including *P. suffruticosa*, *P. lactiflora*, and *P. obovata*, have been used in traditional Chinese medicines and herbal medicines in China and Japan.[22,25] Paeoniflorin, isolated from *P. lactiflora*, is a predominant component of peony plants. Peony extracts and their constituents have various biologic and biomodulating activities, including antioxidant activity, improvement of memory, antiepileptic activity, antimutagenic properties, and antihyperglycemic effects.[12] However, until the current study, whether paeoniflorin exerted any renoprotective effect in diabetes was unknown. To address this question, we evaluated the effect of paeoniflorin on urinary albumin excretion in diabetic rats. Urinary albumin has been suggested as a predictive indicator for prognosis of diabetic nephropathy, and its elevated excretion is said to impair renal function.[4] Furthermore, reductions in urinary albumin in diabetic nephropathy reportedly were associated with renal protection.[15] Here we show that diabetes caused significant increases in the urinary albumin:creatinine ratio, whereas paeoniflorin suppressed this effect (Table 1).

Although the pathogenesis of diabetic nephropathy is multifactorial, tissue fibrosis is a key pathologic hallmark of this disease, in which buildup of ECM proteins is a central pathogenic process.[13] In the present study, experimentally induced diabetes in rats was associated with increased expression of type IV collagen, but paeoniflorin normalized these diabetes-induced elevations in type IV collagen proteins (Figures 2 and 3). Increased glomerular levels of TGFβ reportedly contribute to the pathogenesis of diabetic glomerular lesions.[11] Here, we have shown that the increased TGFβ levels in diabetic nephropathy were suppressed by paeoniflorin. In our study, paeoniflorin treatment reduced activation of the transcription factor NFκB, which is a key factor involved in ICAM1 transcription. Thus, expression of ICAM1 was inhibited also, leading to reductions in the number of macrophag-

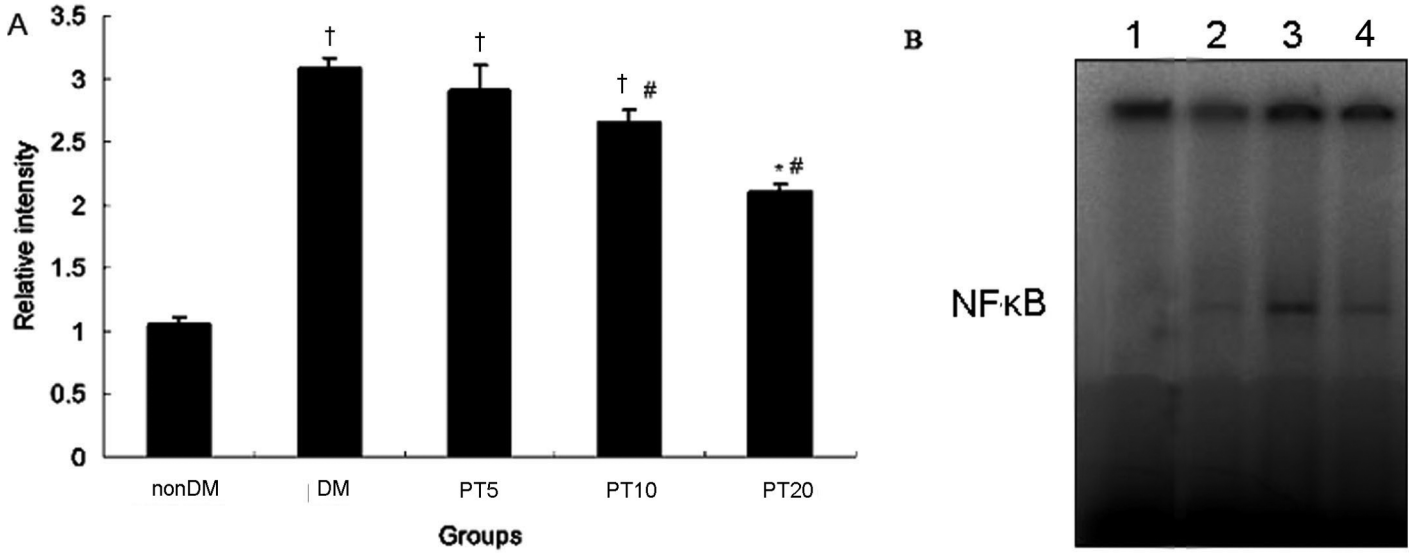

Figure 7. (A) NFκB activity in the renal cortex of nonDM, DM, and paeoniflorin-treated (PT5, 5 mg/kg daily; PT10, 10 mg/kg daily; PT20, 20 mg/kg daily) rats. *, $P < 0.05$ compared with value for nonDM rats; †, $P < 0.01$ compared with value for nonDM rats; #, $P < 0.05$ compared with value for DM rats; and +, $P < 0.01$ compared with value for DM rats by 1-way ANOVA with post hoc Tukey tests. (B) Electrophoretic mobility shift assay for NFκB activation in kidneys of rats. Lane 1, unlabeled oligonucleotide (negative control); lane 2, nonDM rats; lane 3, DM rats; lane 4, PT20 rats.

es recruited into diabetic glomeruli and in the expression of TGFβ and type IV collagen. TGFβ is a key cytokine in the pathway underlying tissue fibrosis and can be activated by multiple upstream effectors.[16,19] Its reduction during the diabetic state may be due to decreased numbers of macrophages, because macrophages themselves are known to secrete TGFβ and to stimulate mesangial cells to produce TGFβ.[26]

In summary, we have shown here that the protective effects of paeoniflorin treatment on diabetic nephropathy may be mediated by its antiinflammatory actions, including inhibition of TNFβ, type IV collagen, and ICAM1 expression; NFκB activation; and renal macrophage infiltration.

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